IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Dan Nilsson and Thomas Janzen.
Serial No.:	09/720,096
Filed:	December 21, 2000
For:	Method of preventing bacteriophage infection of bacterial cultures
Examiner :	Steadman, D. J.
Group art unit:	1652

Declaration of Dan Nilsson

I, Dan Nilsson, having my residence at Gefionsbakken, 4A, DK-3060 Espergærde, Denmark, does state and declare as follows:

1. I am an employee at Chr. Hansen A/S, the assignee of the above patent application, and I hold a position as Director of Innovation, M.Sc. , Ph. D.

2. I believe that I am a person skilled in the art to which the above application pertains.

3. I have read and understood the pending claims in that application as well as the office action related thereto dated March 27, 2002 as well as the cited documents and have the following comments:

4. I have carried out a study to demonstrate that the use of milk as a substrate material as claimed in the above application is just for illustrative purposes and that the illustration should not be interpreted as a limiting feature of the claims.

The following study shows that the invention performs equally well in the medium M17, described by Terzaghi & Sandine 1975, and the medium LSA, described by, Jensen & Hammer 1993, as in milk. In these media, the mutant we tested was *Lactococcus lactis* MBP71 (*thyA*) from example 2 of the present patent application. These studies show that the bacterium is metabolically active even though it is blocked in DNA replication independently of the substrate material used.

The results of the study are summarized in the following section (5):

5. Test for growth and metabolic activity for *Lactococcus lactis* strain MBP71 in different mediums.

Milk as substrate material

For MBP71 inoculated into milk the CFU did not increase (example 2 of the present invention) as CFU would do for a normal growing strain. Hence, the lack of increase in the CFU for MBP71 growing in milk is due to the blockage of DNA replication. Under these conditions of non-replicating growth, the metabolic activity of the cells, i.e. the acidification, is completely insensitive to the presence of phages.

M17 medium as substrate material

In respect of the substrate material M17 we have shown that the CFU does not increase in this medium either. In fact the CFU showed practically the same behavior as in milk. This shows that DNA replication is also blocked in this medium. At the same time, the optical density at 600nm (OD600) of the culture increased linearly as opposed to exponentially when thymidine was supplemented, or when a normal growing culture was tested, i.e. the mother strain of MBP71, CHCC373. This establishes that the cells are metabolically active. And to further substantiate this, we have shown that MBP71 also acidifies M17 in a manner similar to milk (see Fig. A), i.e. the cells are metabolically active.

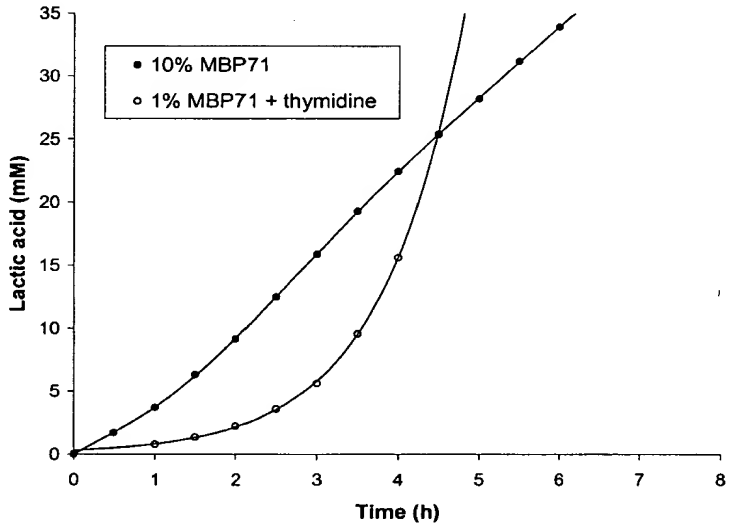

Fig A: Acidification of LM17 by MBP71 with and without thymidine.

Stationary cultures of MBP71 in LM17 (M17 + 0.5% of lactose) with thymidine at 30°C were washed and inoculated into LM17 with or without thymidine and pH was followed over time. As the pH of M17 decreases linearly from pH 6.8 to 5.8 as acid is added (37.7 mM/pH unit), the pH values were converted into amount of lactic acid formed as shown in the graph. The inoculation levels are designated as in the patent.

SA medium as substrate material

An experiment similar to the one carried out in M17 was carried out in LSA medium (SA medium + 1.0% of lactose). The growth curves for MBP71 in LSA with or without thymidine are shown in Fig. B. the study showed that MBP71 grows exponentially in SA medium when thymidine is supplemented whereas MBP71 grows linearly in SA medium when thymidine is absent. As for M17 this shows that DNA replication is blocked, but that the cells are still metabolically active.

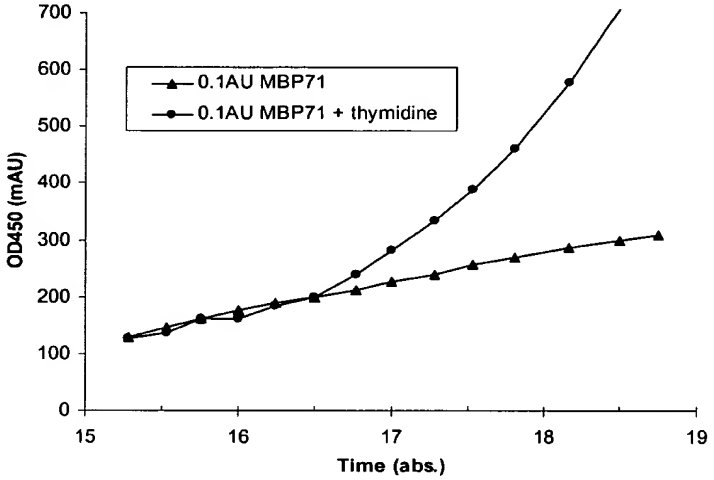

Fig. B: OD450 in LSA of MBP71 with and without thymidine.

Cultures of MBP71 growing at 30°C in LSA supplemented with thymidine were inoculated into fresh LSA with thymidine. After 5.5 hours (about OD450=1.0AU, absorbing units) the cells were harvested, washed, and inoculated at 0.1AU into fresh LSA with or without thymidine. Then OD450 was followed over time as shown in the graph.

Conclusion

We have shown in great detail how the DNA replication of MBP71 is blocked in both 1) milk, 2) complex M17 medium, and 3) defined SA medium, i.e. in three substantially different substrate materials, unless the nucleoside thymidine is supplemented. We have shown that MBP71 acidifies both 1) milk and 2) M17 and also increase in OD in 3) SA medium, which must also lead to acidification, i.e. MBP71 is metabolically active in all these media. In summary, a strain such as the one described in Claim 1, e.g. MBP71, growing in various substrate materials, is blocked in DNA replication, is metabolically active, and is completely insensitive to bacteriophages.

Thus, based on the examples shown here and in the present invention, the bacteria strains listed on page 18, lines 21-28, of the specification and the substrate materialas listed on page 10, line 29, to page 11, line 3, of the specification, I could make use of the entire scope of the present invention.

6. As reported herein, the experiments have been carried out in accordance with good laboratory practice and in the same manner as example 2 of the present invention.

7. I further declare that all the statements made herein of my own knowledge are true and that the statements were made with knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardise the validity of the application or any patent issued thereon.

Dated: 26 July 2002 Signature: _____

Dan Nilsson